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RECOVERY OF CARBAPENEM RESISTANT ENDOMETRIAL BACTERIAL ISOLATES FROM POSTPARTUM ENDOMETRITIS IN DAIRY CATTLE

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ABSTRACT

In this report, we describe recovery of multiple drug resistance organism resistant to most of the antibiotics with coexistence of ESBL, Carbapenam and AmpC ß-lactamase genes isolated from the bovine clinical endometritisin the Mathura region of India.Sampling involvesuterine discharge collected from 70 postpartum cattle diagnosed with metritis. Out of 62 bacterial isolates recovered, seven were showing carbapenamase resistant phenotype baseddisc diffusion test. MIC testing and phenotypic detection methodconfirmed resistance to imepenam for isolates of *Pseudomonas aeruginosa (n=1),E. coli* (n=2) and *Enterobacter agglomerance (n=1)*. Multiplex PCR based genotyping confirmed presence Ambler class B Carbapenemase (VIM and IMP), along with TEM and CTX-M type ESBL respectively. All four isolates were phenotypically confirmed as AmpC β-lactamase producers.

Key words: CR-GNB, AmpC betalactamase, Endometritis, bla VIM, bla IMP

Introduction

Metritis is an infectious reproductive disease of dairy animals, with incidence rate of 20 to 40 percent and most commonly occurs within the first 21 days postpartum. The disease has significant economic implications for the dairy industry due to decreased reproductive performance, delayed restart of estrous cyclicity, and higher risk of culling (Giuliodori *et al.,* 2013).In a clinical setting, failure of antimicrobial therapy to achieve clinical cure in dairy animals with metritis is quite common. Earlier studies pinpoint higher frequency of occurrence of extended-spectrum β-lactamases (ESBLs) producing Gram-negative bacteria from cattle with

postpartum uterine infections (Agrawal *et al.,* 2021). Infections caused by Gram-negative bacteria that produce extended spectrum beta-lactamases (ESBL) are routinely treated with carbapenems, which have a wide range of antibacterial action (Jacoby *et al.,* 2004; Livermore 2001). Based on World Health Organization (WHO) AWaRe (Access, Watch, and Reserve) criterion 2017, carbapenems are classified under 'watch group' and are not yet approved for use in animals. Nevertheless,recent emergence of resistance to carbapenam by strains producing carbapenem-destroying β-lactamases (carbapenemases) has seriously dented its reputation as most reliable last-resort treatment for bacterial infections(Patel and Bonomo, 2013).The occurrence of Carbapenem-resistant Enterobacterales (CRE) in livestock faecal sample and environment has been reported by several researchers globally (Schwaiger *et al.,*2008; Singh *et al.,* 2012, Nandi *et al.,*2013,Ozo *et al.,*2016; Hernandez *et al.,* 2002), as well as in India,(Ghatak *et al.,*2013, Pruthvishree *et al.,*2017, Nirupama *et al.,*2018, Arun *et al.,*2022). However, information regarding their prevalence in uterine infection is completely lacking. Here, we describe detection and characterization of carbapenemase producing Gram-negative bacilli (CR-GNB) in cattle with clinical endometritis.

MATERIAL AND METHODS

1. Sampling

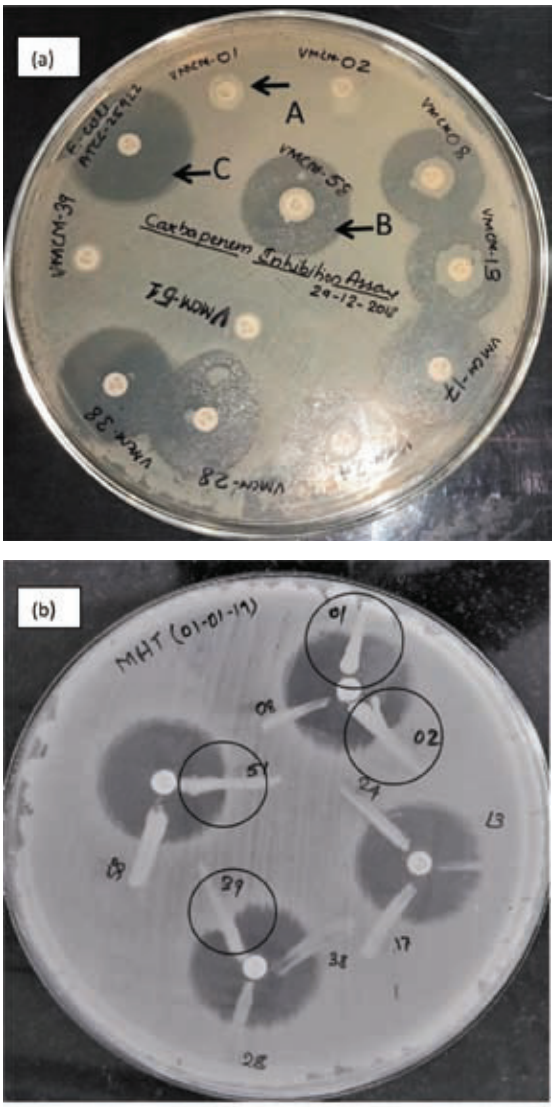
The study includes cows (n = 34) and buffaloes (n = 36), presented to Veterinary Clinical Complex, COVSc & AH, DUVASU Mathura, with a history of clinical endometritis. Uterine discharge was collected by a double-guarded method to prevent vaginal contamination and processed in the laboratory for the isolation of bacterial organisms. The collected samples were pre-enriched in buffered peptone water.

2. Isolation and Identification of bacterial strains:

Initial screening was done by growing a pre-enriched sample on MacConkey agar supplemented with cefotaxime and ceftazadime at a concentration of 2 µg/ml. Primary growth was subcultured on a MacConkey agar plate supplemented with both cefotaxime and ceftazadime at a final concentration of 1 ug/ml. Purified isolates were identified using the procedures described by Cowan and Steel. (Barrow and Feltham 1993).

3 Antibiotic susceptibility test

An antimicrobial susceptibility test was performed for all the isolates by the standard Kirby-Bauer disk diffusion method using Mueller-Hinton agar



(Sigma Aldrich, USA) according to CLSI recommendations (CLSI-M100-S27).The panel of antimicrobial agents consisted of10different Antimicrobial-impregnated disk (BD BBL, Sensi-Disc) namely Ertapenem (10 µg) , Cefotaxime(30 µg) , Cetazidime (30 µg) , Gentamicin (10 µg) , Ampicillin (10 µg) , Amoxicillin-clavulanate(10 µg) , Ciprofloxacin(5 µg) , Cefoxitin(30 µg) , Ceftriaxone(30 µg), Cefpodoxime (10 µg). Zone diameter was measured and interpreted as per the Clinical and Laboratory Standards Institute (CLSI, 2017) guidelines.*Control strain includes Klebsiella pneumoniae ATCC BAA 1705*$^{+KPC}$and *Klebsiella pneumoniae ATCC BAA 1706.*

4. Phenotypic detection of carbapenemase production: All the isolates suspected of producing carbapenemase based on zone diameter breakpoints corresponding to resistant phenotypes were included in

Figure 1 a-c : (a) Carbapenem inactivation method (CIM) test result of selected isolates. Test isolates showing positive results (A) with absence of an inhibition zone while negative results (B) is indicated by appearance of □20 mm of inhibition zone diameter. (b) Modified Hodge test result of selected isolates. Positive test characterized by cloverleaf indentation. (c) Modified Carba NP test results test result of selected isolates (a) Tube containing phenol red solution 0.1 mM $ZnSO_4$ (pH 7.5 (b) Tube containing phenol red solution 0.1 mM $ZnSO_4$(pH 7.5) supplemented with 6 mg/mL of imipenem

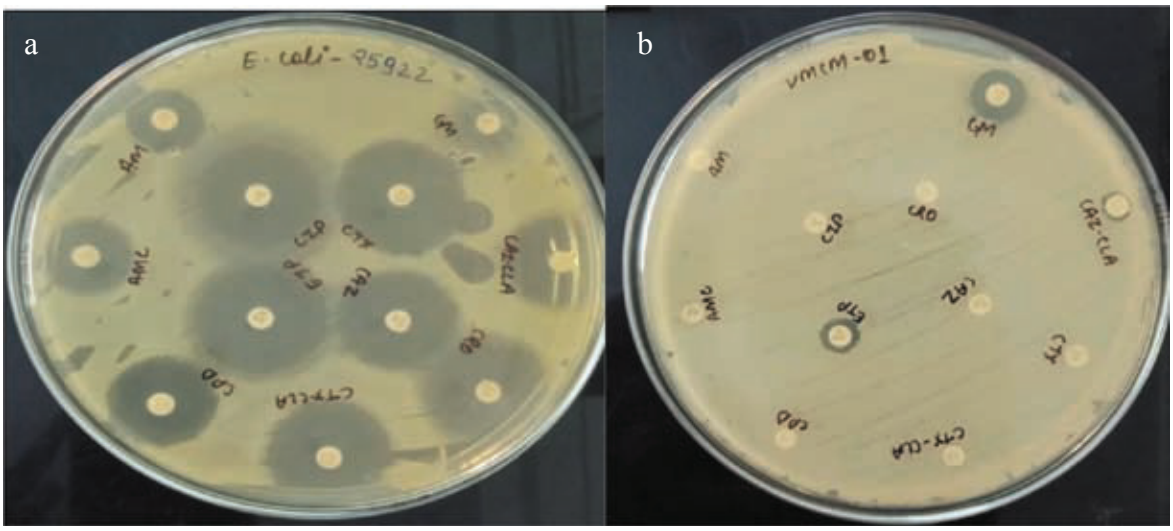


Figure 2 : Showing result of antimicrobial susceptibility test by disc diffusion method. (a) *Eschericha coli* ATCC 25922 (b) ABST result of representative test isolate

the study. All the CRE suspected isolates were grown on Trypticase soy agar for an overnight incubation prior to phenotypic testing, the modified Carba NP test (Rudresh *et al*., 2017), the Carbapenemase Inactivation Assay (CIA) (van der Zwaluw *et al*., 2015), and the modified Hodge test (MHT) (Amjad et al., 2011), as previously described. The AmpC disk test was carried out according to the procedure described by Black *et al*. (2005). *E. coli* ATCC 25922 is used as a standard.

5. Minimum Inhibitory Concentration (MIC): All the strains found resistant to imipenem during the disk diffusion test were tested for MIC determination by the broth microdilution method in accordance with the 2017 guidelines of the CLSI (CLSIM100-S27). Three to four well-isolated colonies from an overnight-grown culture plate were transferred to sterile saline with a loop. The suspension is adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard. The bacterial suspension, which was so adjusted to have 1 $X10^8$ $cfuml^{-1}$, was further diluted by a factor of 1:100 so as to achieve approximately 5 × 105 cfuml-1in the final test concentration of the bacteria. The test concentration of imipenam was prepared by dissolving 25.6 mg of imipenam-cilastin injection in 10 ml of sterile distilled water (equivalent to 12.8 mg of imipenam). Working antibiotic stock solution was prepared by a 1:10 dilution of antibiotic stock solution in Muller Hinton Broth (MHB). The plates were covered by sterile covers and incubated at 37°C for 18–24 h. The lowest concentration of the antibiotics that did not have visible bacterial growth was defined as the MIC. *Escherichia coli* ATCC 25922 was used as a quality control strain.

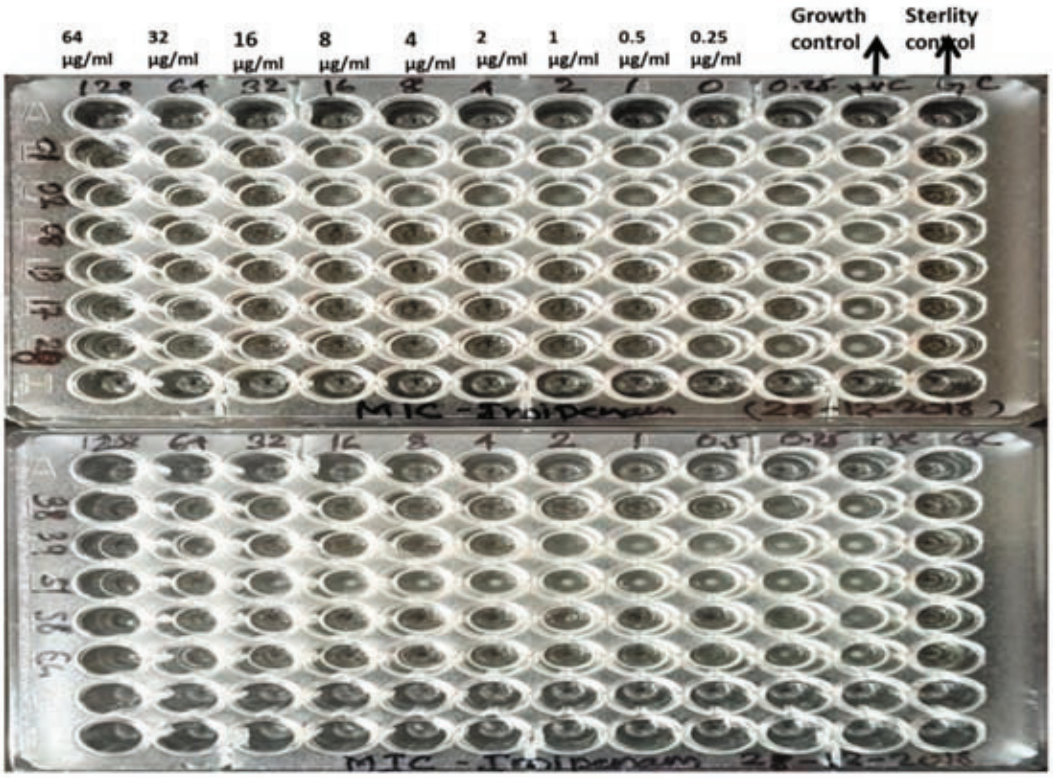


Figure 2: Broth microdilution plate for detection of Imipenem MIC in representative isolates; A12 to H12 are negative growth control wells. A11 to H11 wells are positive growth control wells.

6. Analysis of anti-microbial resistance genes: DNA isolated by snap chill method was subjected to a target amplification of CRE and ESBL associated genes encoding *bla*OXA-48, *bla*IMP, *bla*VIM and *bla*KPC, *bla*CTX-M, *bla*TEM and *bla*SHV using previously described oligonucleotide primers (Dallenne *et al*., 2010). A 25µl reaction mixture containing 12.5 µl Dream Taq Master mix, 1.25 µl of primers and 2 µl of isolated DNA template.Amplification was carried out as follows:initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40s, 55°C for 40s, 72°C for 1 min and the final elongation step at 72°C for 7 min.

RESULT

A total of 62 bacterial strains were isolated and identified by biochemical tests into six different genera: *Escherichia coli*, *Klebsiella spp*., *Pseudomonas spp*., *Enterobacter spp*., *Citrobacter spp*., and *Serratia spp*. On the basis of decreased susceptibility to Ertapenem upon antimicrobial susceptibility tests, seven isolates were selected as suspected CR-GNB having a zone diameter less than the breakpoint. The resistant isolates were tested for carbapenamase production by phenotypic methods, viz., MHT,

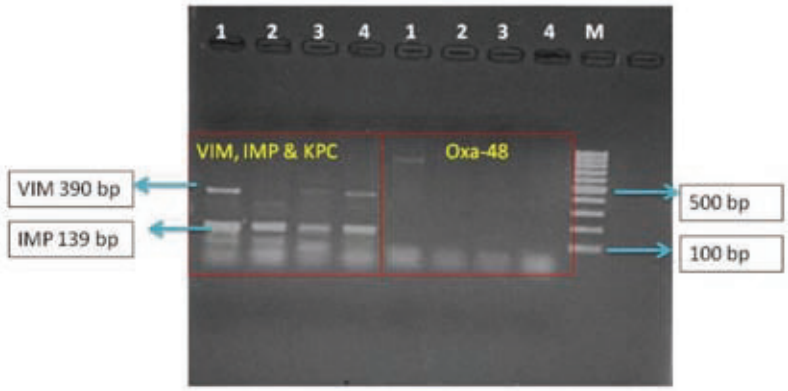


Figure 4: PCR amplification of bla VIM, bla IMP, blaKPC and blaOxa-48 gene . Lane M: 100 bp DNA ladder, Lane 1,2,3,4: carbapenam resistant isolates

m-CARBA-NP, and CIA tests. (Figure 2). All four isolates were positive for all three phenotypic confirmatory tests as well as the AmpC disc test. The antibiogram showed that the isolates were resistant to cefotaxime (100% each), cefpodoxime (100%), ceftriaxone (85.71%), and ceftazidime (71.4%) (Figure 1). Isolates were observed to be resistant to penicillin, cephalosporin, and fluoroquinolone antimicrobials, including carbapenems, but remained susceptible to aminoglycosides. The MIC for Imipenam for all seven CRE isolates ranged from 0.05 ug/ml to 64 µg/ml. The MICs of all four CP strains were ≥35 µg/mL (Table 1, Figure 3). Multiplex PCR analysis identified the blaIMP gene in all four CRE isolates, while three of them carry the blaVIMgene (Figure 4). None of the isolates were found positive for bla KPC or blaOxa48. Two isolates also exhibited the presence of CTX-M and TEM-type ESBL genotypes (Figure 5).

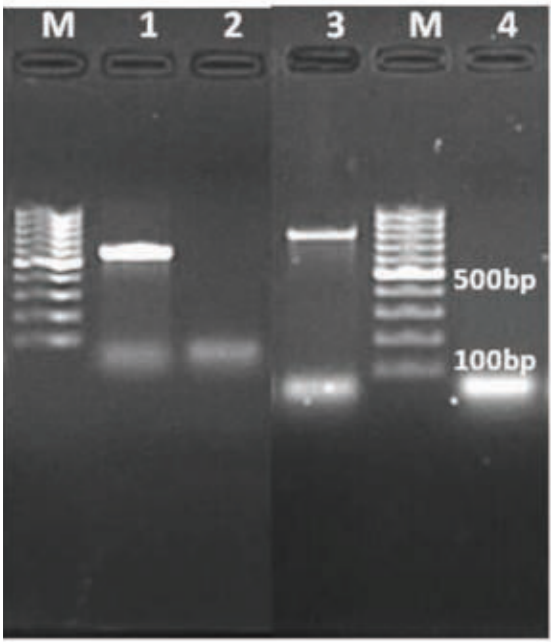


Figure5 : PCR amplification of bla CTX-M and bla TEM gene. Lane M: 100 bp DNA ladder, Lane 1: Isolate showingspecific amplification of bla CTX-M,Lane 3: Isolate showing specific amplification of bla TEM, Lane 2 and 4: E. coli ATCC 25922

DISCUSSION

This study reports the occurrence of carbapenem-resistant organisms from bovine clinical endemetritis. The isolated Carbapenem-Resistant Gam-negative

Table 1: Details of Primers used in the study

Primer Name	Sequence (5'-3')	Amplicon Size	Reference
CTX-M _F	CGA TGT GCA GTA CCA GTA A	580	Hopkins *et al* 2007
CTX-M_R	TTA GTG ACC AGA ATC AGC GG		
TEM_F	CATTTCCGTGTCGCCCTTATTC	800	Dallenne *et al;* 2010
TEM_R	CGTTCATCCATAGTTGCCTGAC		
SHV_F	AGCCGCTTGAGCAAATTAAAC	713	
SHV_R	ATCCCGCAGATAAATCACCAC		
OXA-48_for	GCTTGATCGCCCTCGAT	281	
OXA-48_rev	GATTTGCTCCGTGGCCGAAA		
bla IMP-F	TTGACACTCCATTTACDGa	139	
bla IMP-R	GATYGAGAATTAAGCCACYCTa		
bla VIM-F	GATGGTGTTTGGTCGCATA	390	
bla VIM-R	GATGGTGTTTGGTCGCATA		
bla KPC-F	CATTCAAGGGCTTTCTTGCTGC	538	
bla KPC-R	ACGACGGCATAGTCATTTGC		

bacilli (CR-GNB) strains were carrying coexisting carbapenases (blaVIM, blaIMP), CTX-M beta lactamse, and AmpC beta lactamase. Carbapenems are potent antimicrobial agents for the treatment of various infections in humans caused by Gam-negative bacilli (GNB) resistant to practically all alternative antibiotics (Livemore, 2007). A global blanket ban and absence of products for animal use that contain carbapenems completely exclude their use in livestock; hence, carbapenem-resistant bacteria appear to be rare in cattle. However, in recent years, several workers across the globe have detected CR in livestock, companion animals, and their environment (Kluytmans *et al*., 2013; Guerra *et al*., 2014).We observed a 6.45 percent prevalence of CRE organisms in our study. Köck *et al*. (2018) inferred a low prevalence of CR-GNB among livestock (<1%) in European countries and a higher prevalence in Asia based on the systematic review of all studies published in the PubMed database. In India, in a recent study, 29.03 percent of isolates were resistant to at least one of the three carbapenems tested (Murugan *et al*., 2019). We have recovered enterobacteria (*E. coli*, *Enterobacter*, *Serratia*) and Pseudomonas, from the infected uterus in the present study. These organisms are known to carry the carbapenam-resistant gene as well as being the most common colonizers of the intestine. The colonization of CR-GNB strains in the uterus may possibly be

Table 2: Details on the tests performed using different CR-GNB isolates

Isolate No	Species	m-Carba test	CIA	Modified Hodge test	AmpC Disc Test	MIC (Imipenam) observed S≤1 µg, I 2-4 µg, R ≥4 µg	CTX-M	TEM	SHV	VIM	IMP	KPC	Oxa48
VMCM-01	*Psudomonas*	+	+	+	+	32 µg	-	-	-	+	+	-	-
VMCM-02	*E. coli*	+	+	+	+	32 µg	-	-	-	-	+	-	-
VMCM-13	*Enterobacter agglomerans*	-	-	-	-	0.5µg	+	-	-	-	-	-	-
VMCM-21	*E. coli*	-	-	-	-	1 µg	+	-	-	-	-	-	-
VMCM-28	*Serratia*	-	-	-	-	1 µg	-	-	-	-	-	-	-
VMCM-39	*Enterobacter agglomerans*	+	+	+	+	4 µg	+		-	+	+	-	-
VMCM-51	*E. coli*	+	+	+	+	64µg	-	+	-	+	+	-	-
Negative control	*E. Coli ATCC 25922*	-	-	-	-	0.5µg	-	-	-	-	-	-	-

attributed to postpartum fecal contamination of the uterus, which remains the most remarkable cause of clinical endometrilitis. The CR-GNB strains examined in this study were resistant to different classes of antimicrobial agents, mainly carbapenems, antipseudomonal penicillins, quinolones, and cephalosporins, including carbapenems, and were therefore considered MDR. All four GNB isolates were positive for the phenotypic AmpC β-lactamase detection test. All were resistant to co-amoxiclav, ceftazidime, cefotaxime, ceftriaxone, and cefoxitin. Current findings are in accordance with the work done by other researchers (Ding *et al*., 2008; Park *et al*., 2010). AmpC β-lactamases confer resistance to aminopenicillins, carboxypenicillins, ureidopenicillins, and cephalosporins, as well as extended-spectrum cephalosporin β-lactamase inhibitors like clavulanic acid (Jacoby, 2009). The MIC of imipenam (≥35 µg/ml) observed in the present study in the CRE isolates was in concordance with earlier reports (Shanthi *et al*., 2014; Pruthvishree *et al*., 2017).

Carbapenemase-encoding genes have been reported across a variety of Enterobacteriaceae genera (Kochar *et al*., 2009). The Molecular Classification of Carbapenemase Enzymes categorizes it predominately into five major classes represented by the KPC, OXA-48-like, NDM, VIM, and IMP families (Mathers et al., 2015). There are few reports of animal carriage of blaVIM-1 isolates around the world, including a recent report

of the isolation of blaVIM-1-carrying *E. coli* isolates from diarrheal calves in India (Fischer *et al*., 2012; Fischer *et al*., 2013; Murugan *et al*., 2019). All four CP isolates characterized in this report are shown to carry the bla VIM gene. Isolation of a blaIMP-carrying isolate in multiple species of Gram-negative bacteria was reported from a dog in China (Wang et al., 2014), from pigs in the USA (Mollenkopf *et al*., 2018), and from cats in Australia (Abraham *et al*., 2016). To the best of our knowledge, this is the first report of the blaIMPcarbapenemase gene in bovines in India. In the present study, seven isolates showed decreased susceptibility to ertapenam, but confirmatory tests revealed only four isolates as CRE. It is assumed that mechanisms other than carbapenam production are responsible for such findings. Carbapenem resistance in Enterobacteriaceae is mediated either by carbapenemase production, overexpression of efflux pump, or deficiency of bacterial outer membrane protein in combination with a high-level production of AmpC β-lactamase, or ESBL (Nordmann et al., 2011). The case history of the animals included in the present study indicates prior treatment with ceftiofur and oxytetracyclin. Webb and coworkers hypothesized that the selective pressure of ceftiofur use may have favored the dissemination of β-lactamases as well as the carbapenemase gene (Webb et al., 2016). Ceftiofer sodium and oxytetracyclin are most frequently used in the treatment of clinical endometritis; hence, it can also be hypothesized that coadministration of cephalosporin and oxytetracylin may favor the co-selection of the carbapenam-resistant gene. Although the exact role of extended-spectrum cephalosporin use in the development of carbapenem resistance is still obscure, the selection pressure provided by these antimicrobials may favor the emergence and dissemination of a carbapenem resistance phenotype resistant to all extended-spectrum cephalosporins. The consequences of the expansion and dissemination of carbapenem-resistant bacteria in livestock have a significant and far-reaching effect on the therapeutic outcome of the disease. Hence, it is obligatory that continuous surveillance of carbapenem susceptibility among enteric bacteria in livestock be done from a true perspective.

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Competing Interests

The authors declared that they have no competing interests.

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